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ADHESIVE AND NON-ADHESIVE MEMBRANE DOMAINS OF AMPHIBIAN EMBRYO CELLS

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SUMMARY

Superficial blastomeres of mid-cleavage stage amphibian embryos (32- and 64-cell stage) display regional cell-surface differences in adhesiveness. The cells are adhesive on the lateral and basal cell surfaces and non-adhesive on the apical surface. These adhesive differences are maintained by single cells which have been dissociated from the intact embryo. Pigmentation differences afford a means of distinguishing apical surface from basal/lateral surface in dissociated cells. The apical surface is underlain by melanin granules and so appears dark, whereas the lateral and basal surfaces lack pigment and are white. Scanning electron-micrograph observations of isolated superficial cells reveal striking morphological differences between the adhesive and non-adhesive regions. The apical (non-adhesive) portion of the cell surface has a convoluted appearance and is almost entirely devoid of microvilli. The lateral and basal (adhesive) surfaces lack the convolutions of the apical surface and bear scattered microvilli.

The isolated superficial cells continue to divide in culture. The cells display a unilateral or one-sided furrow which begins in the basal surface and progresses toward the apical surface. The plane of cleavage of most of the cells is oriented such that the pigmented cap is bisected, with very few cells having the plane of the furrow pass parallel to the apical surface. The direction of furrowing in the isolated cells from the 32- to 64-cell embryo represents a direction of furrow progression in the intact embryo from the interior to the surface. Since the early cleavages are initiated at the surface and progress into the interior, it is suggested that a reversal in direction of furrowing occurs by mid-cleavage stages.

INTRODUCTION

In work on the isolation, behaviour and aggregation properties of embryonic amphibian cells Holtfreter (1943*a*, *b*, 1944, 1947) described a cell type, the superficial cell, which maintains regional differences in adhesiveness, which comprises the outermost cell layer of the embryo and which is non-adhesive to other cells and to artificial substrata over that part of the surface which faces the outside of the embryo (the apical surface) but is adhesive on lateral and basal surfaces. It is well known by experimental embryologists that 2 embryos placed in contact will not adhere if the only contact is between apical cell surfaces. Adhesion occurs readily if the embryos are wounded slightly and the wounded areas are apposed, allowing contact between lateral cell surfaces (Hamburger, 1960; Holtfreter, 1943*a*, p. 283; Rugh, 1962). The superficial cells maintain these regional differences in cellular adhesiveness even after dissociation from the intact embryo (Holtfreter, 1943*a*, *b*, 1944, 1947). When dissociated superficial cells are allowed to reaggregate *in vitro*, they orient themselves

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in the aggregates with the pigmented portions of the cell surface at the surface of the aggregate, re-establishing the polarized organization of the intact embryo. The adhesive and non-adhesive cell surface regions can be distinguished on dissociated cells by the presence of cortical pigment granules which underlie only the non-adhesive cell surface region.

The maintenance of circumscribed non-adhesive domains is characteristic of epithelia and may be important in maintenance of the epithelial character of these tissues (Elsdale & Bard, 1974; Okada, 1965). Likewise, the maintenance of the internal lumens of blood and lymphatic vessels, kidney tubules, excretory and reproductive ducts, the gut and other derivatives of the endoderm may depend on the preservation of a non-adhesive character to the apical surfaces of the cells lining these structures (Holtfreter, 1943*a*, *b*, 1944). Although the mechanism of cell–cell adhesion has received considerable attention, cellular non-adhesion, although potentially of considerable physiological importance, has received little attention. The amphibian embryo superficial cells are useful subjects for study of non-adhesiveness since the cells are large, the non-adhesive domain is preserved in dissociated cells in culture and the non-adhesive plaque can be identified in the living cell by the cortical pigment marker.

In an attempt to increase our understanding of the mechanisms involved in cellular adhesiveness and non-adhesiveness, we studied the surface morphologies of isolated superficial cells using the scanning electron microscope (SEM). This study revealed major differences between the surface morphologies of the adhesive and the non-adhesive membrane regions. Of particular interest was a strong correlation between the presence of microvilli on a cell surface region and the ability of that region to adhere to other cells. The non-adhesive surface region was a contracted knob with essentially no microvilli, while the adhesive region had numerous microvilli. The junction between these 2 regions was characterized by a dense array of microvilli.

MATERIALS AND METHODS

Rana pipiens eggs were procured by hormonally induced ovulation and fertilized *in vitro* (Wright & Flathers, 1961). At mid-cleavage stages (32- to 64-cell stage), the jelly coats were removed manually with sharpened watchmaker's forceps and the embryos were dissociated by the method of Holtfreter (1943*b*). The pH of the media was raised to 10·2 by the addition of KOH. After approximately 10 min the embryo was dissociated into a mass of single cells. The vitelline envelope was removed with watchmaker's forceps resulting in release of the embryonic cells. The superficial cells of the animal hemisphere were sorted with hairloops from the cells that in the intact embryo lay deep to the surface, and removed to another dish of complete Holtfreter's solution (pH 7·6) with a Pasteur pipette. All procedures were done in Petri dishes coated with 2 % agar to prevent adhesion of the cells to the surface of the dishes.

For observation with the SEM, the superficial cells were fixed at room temperature for 24 h in a mixture containing 2 % paraformaldehyde, 2 % glutaraldehyde, 0·5 % acrolein with 2 mM $CaCl_2$ in 0·1 M sodium cacodylate buffer. Cells were washed in 0·1 M cacodylate, post-fixed for 1 h in 1 % osmium tetroxide in cacodylate buffer, dehydrated in ethanol, rinsed with amyl acetate, and dried in a Bomar critical point drying apparatus. The dried cells were mounted on stubs with double-sticky Scotch-tape, coated with evaporated gold–platinum and observed with a Philips Model 501 SEM at an accelerating voltage of 15 kV.

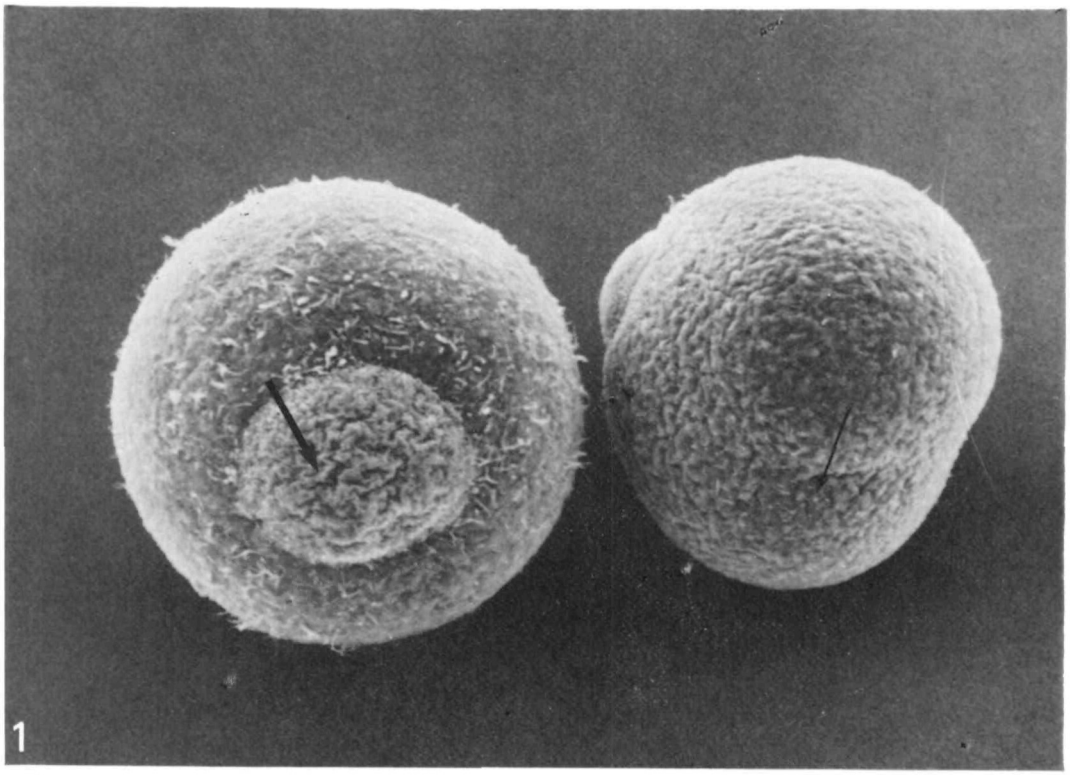
RESULTS

Mid-cleavage stage *Rana pipiens* embryos are readily dissociated into viable populations of intact cells 100–300 nm in diameter by brief treatment at high pH. Cells disaggregated by brief treatment at high pH are very adhesive when returned to saline at pH 7·6 and reaggregate completely within 10–15 min. In the aggregates that form, the superficial cells present the pigmented surfaces to the exterior, and adhere along the unpigmented surfaces. To our surprise, cells disaggregated with 2 mM EDTA in Ca^{2+}-free Holtfreter's solution reaggregate more slowly and incompletely than cells disaggregated by high pH treatment. Lower concentrations of EDTA are ineffective in dissociation of the superficial cells of cleavage-stage embryos. Late gastrula/early neurula stage embryo cells, which can be isolated by EDTA concentrations as low as 1 mM, are, similarly, less adhesive than cells dissociated by high pH treatment.

Fig. 1 shows 2 superficial blastomeres from the animal hemisphere which had been isolated from a mid-cleavage stage *Rana pipiens* embryo. A contracted band delineates the border between apical and lateral cell surface regions. The apical (non-adhesive) portion of the cell is distinguishable as a contracted cap with deep convolutions. The size of the cap is highly variable; on some cells it occupies a very small region, while on other cells it may occupy as much as half the cell's surface. Fig. 2 shows a comparison, at higher magnification, of the apical and lateral regions. The apical region is convoluted and displays almost no microvilli, whereas numerous microvilli are dispersed over the lateral region. The lateral region is much smoother, with surface bulges which probably result from yolk platelets lying immediately beneath the plasma membrane. The junction between the apical and lateral surface regions is characterized by a dense, tightly packed array of microvilli (Fig. 3). The length of the microvilli is somewhat variable, but they are most often short and slender as shown in Fig. 3. It remains to be determined if this band of microvilli is present in the intact embryo or if it results from the dissociation procedure. Contraction of this region is presumably a result of dissociation. In the intact embryo this is the region of strongest cell–cell adhesion, since it is the last region to break apart when the embryo is dissociated.

In culture, isolated superficial cells remain mitotically active. When allowed to divide in media which contain Ca^{2+}, aggregates are established by repeated division of single blastomeres. Consistent with Holtfreter's observations on the non-adhesiveness of the apical membrane domains of superficial cells, the pigmented (apical) membrane remains at the surfaces of these aggregates, preserving the polarity shown by the intact cleavage-stage embryo.

During cytokinesis, the isolated superficial blastomeres show an unusual unilateral pattern of furrowing (Fig. 4). The cells in Fig. 4 were cultured in Holtfreter's solution without Ca^{2+} in order to prevent the adhesion of the cells to each other and to facilitate the observation of the progression of the cleavage furrow. The furrow first appeared in the nonpigmented (adhesive) region, nearly opposite the centre of the pigmented region (Fig. 4B). The furrow progressed toward the pigmented region (Fig. 4C),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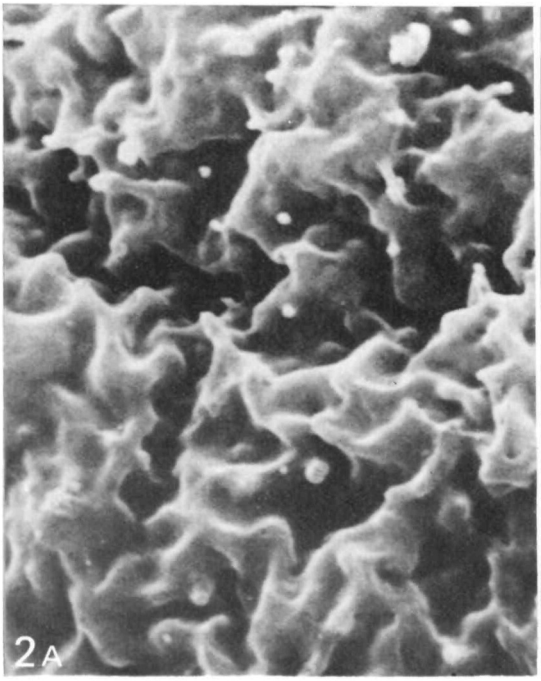
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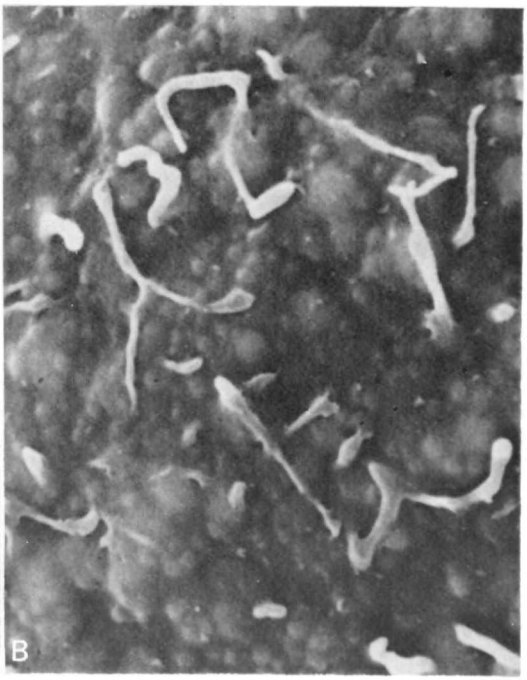
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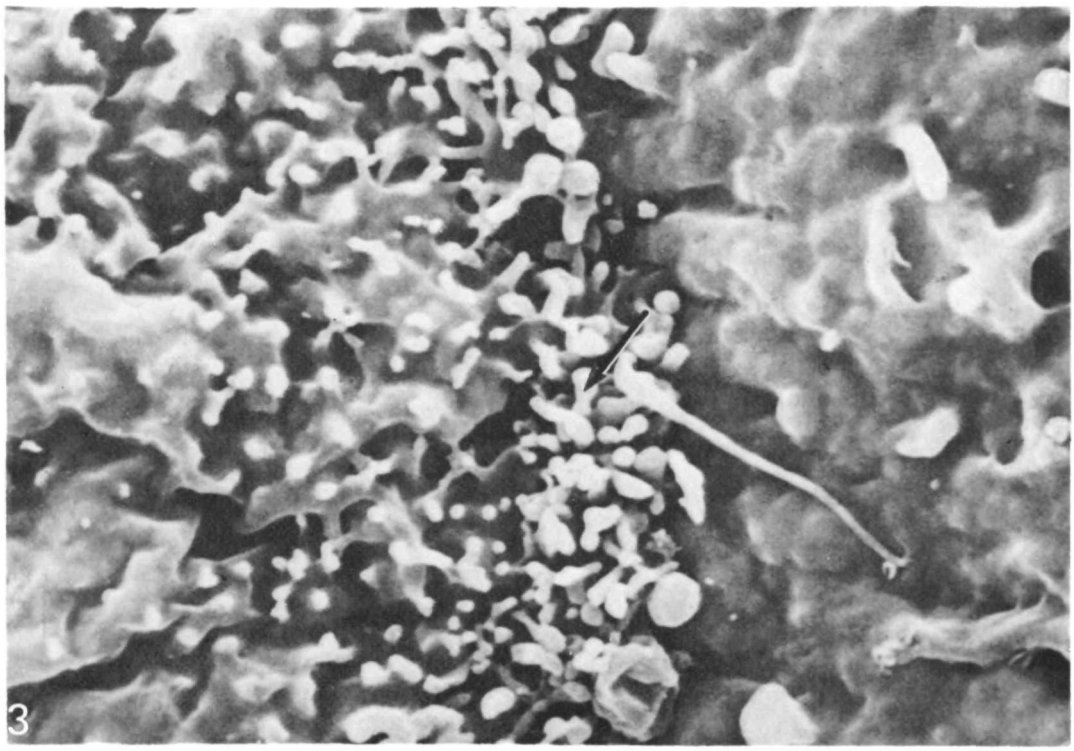

Fig. 3. Scanning electron micrograph of the junction between apical and lateral cell surface regions of a dissociated superficial cell. The region is marked by a dense band of microvilli (arrow). The apical surface is on the left; lateral surface on the right. × 6600.

which did not appear to participate in division until it was contacted by the previously established furrow (Fig. 4D). During cleavage the cell appeared heart-shaped, with an indentation which progressively deepened. Figs. 5–7 are scanning electron micrographs of different stages of cell division.

During the initial cleavage divisions of amphibia, the furrow begins at the surface of the embryo and proceeds into the interior. If the unilateral furrowing pattern observed with the isolated superficial cells is representative of cleavage in the intact mid-cleavage stage embryo, rather than being an artifact resulting from dissociation, then it can be concluded that the direction of furrowing is reversed by the 32- to 64-cell stage, with furrows being initiated at basal cell surfaces and progressing from the interior of the embryo out to the surface. Since microdissection of 32- to 64-cell embryos which had recently initiated a round of cytokinesis revealed unilateral furrowing

Fig. 1. Scanning electron micrograph of dissociated superficial blastomeres from mid-cleavage stage embryo. The convoluted cap (thick arrow) is the former apical surface. The indentation in the second cell (thin arrow) is the beginning of the cleavage furrow in the basal surface. × 480.

Fig. 2. A, apical surface of a superficial cell isolated from a dissociated 32- to 64-cell stage embryo. B, lateral surface of the same cell. × 7500.

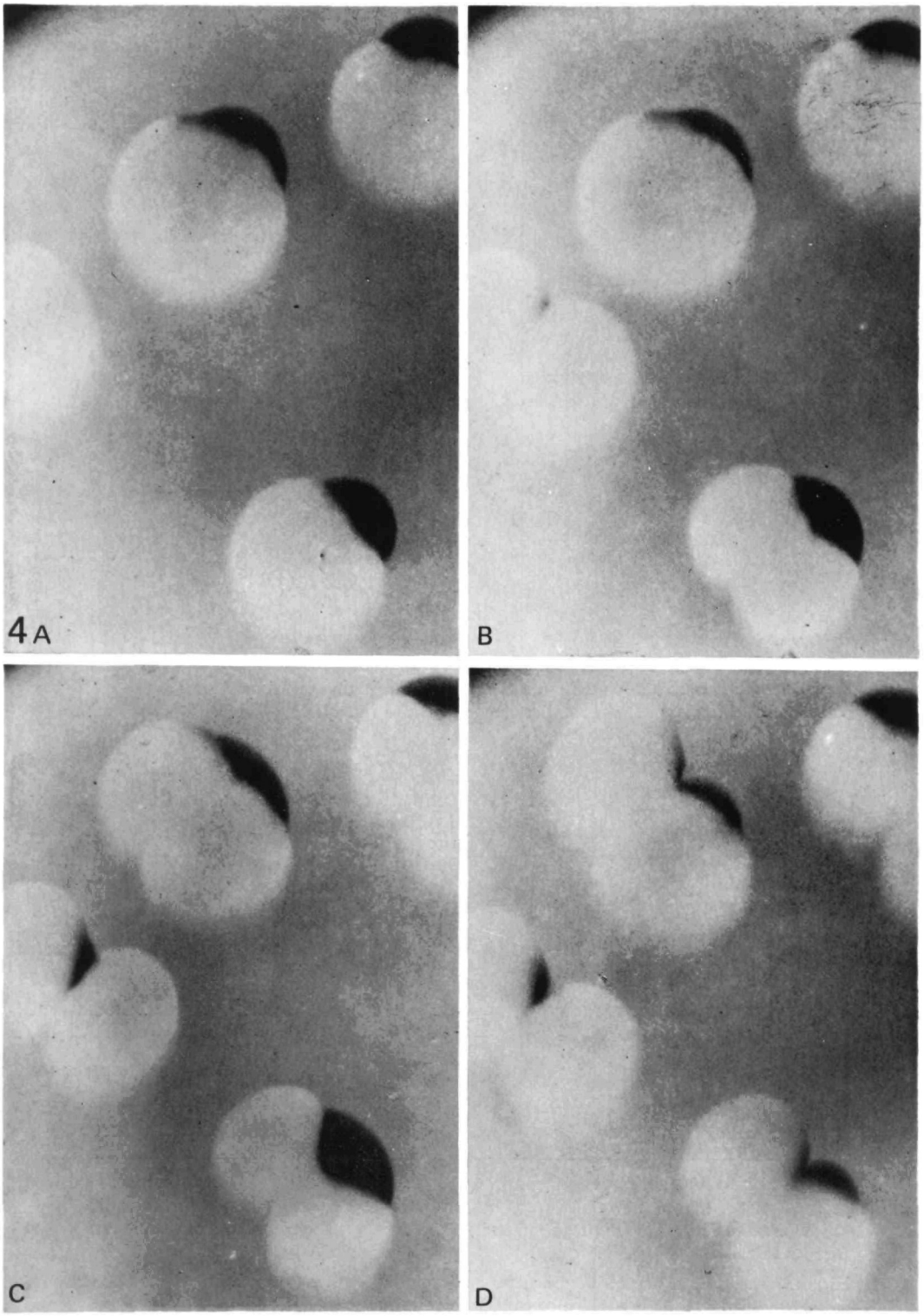

Fig. 4. Sequential views, A–D, of cytokinesis of living isolated superficial cells. The furrow progresses from the basal surface towards the apical surface. × 375.

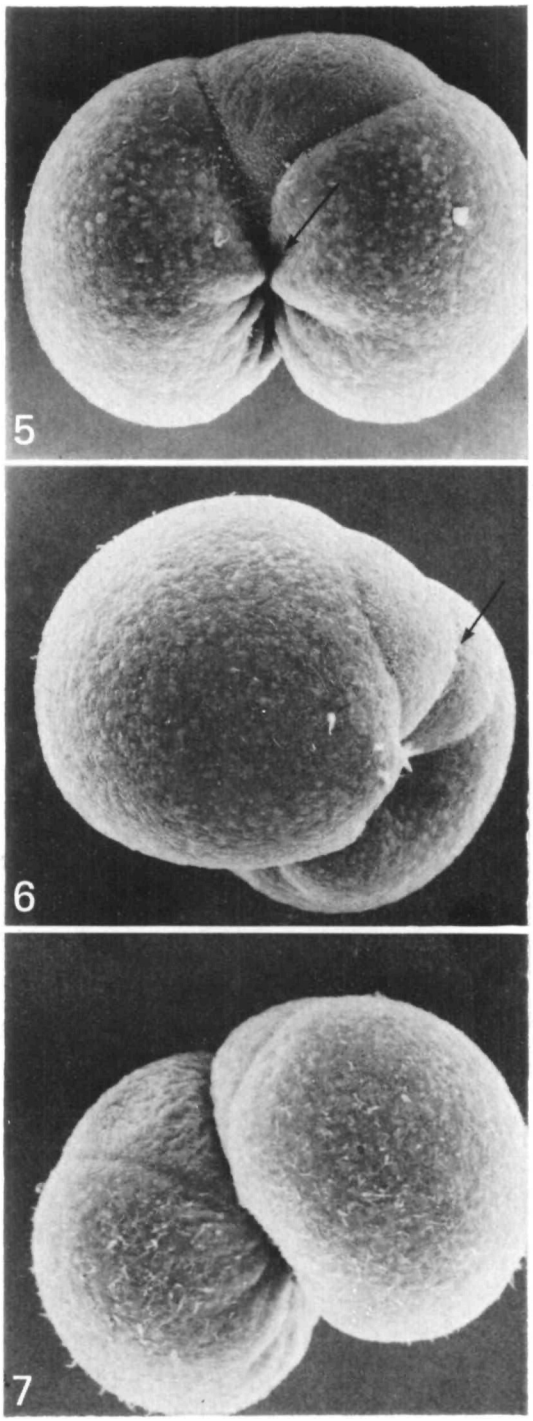

Fig. 5. Scanning electron micrograph of an early stage of cytokinesis of an isolated superficial cell. The cleavage furrow (arrow) has not reached the apical surface. × 520.

Fig. 6. Scanning electron micrograph of a late stage of cytokinesis of an isolated superficial cell. Division of the apical surface (arrow) is beginning. × 520.

Fig. 7. Scanning electron micrograph of the completion of cytokinesis of an isolated superficial cell. The plane of division has bisected the apical surface. × 520.

of the basal surface, it appears that the unilateral basal furrow is characteristic of telophase stage cells both *in vivo* and *in vitro*. Groups of cells which were isolated while in the process of cell division also displayed the unilateral furrowing pattern, suggesting that the pattern observed in isolated cells occurs also *in situ*. If the isolated cells are allowed to continue dividing in media which contain Ca^{2+}, an aggregate of cells is formed which has the same polarity in pigmentation as an intact cleavage-stage embryo.

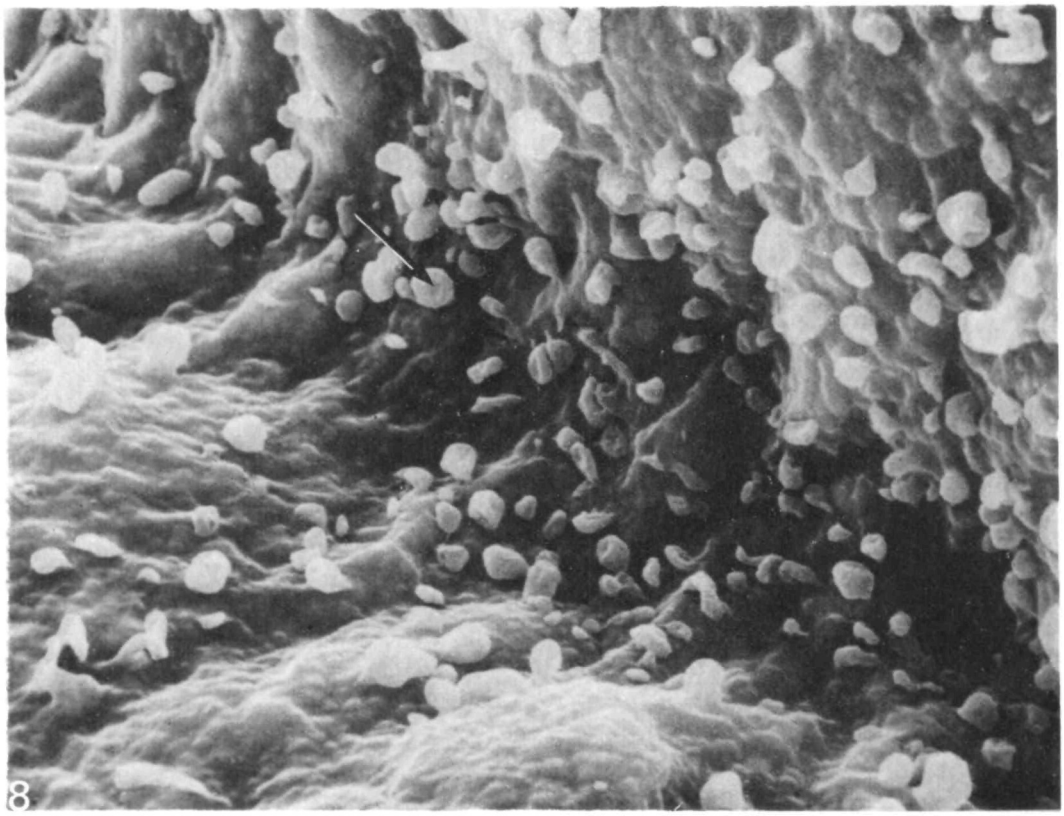

Fig. 8. Scanning electron micrograph of the cleavage furrow region of an isolated superficial cell. An abundance of short, thick surface projections (arrow) is characteristic of this region. × 7500.

Scanning electron micrographs of the cleavage furrow region (Fig. 8) show a concentration of microvilli on the walls of the furrow region. The microvilli are shorter and thicker, in addition to being more abundant, than in the nonfurrow region. An increased number of microvilli in the furrow region of cleavage-stage *Xenopus laevis* embryos has previously been reported by Denis-Donini, Baccetti & Monroy (1976).

DISCUSSION

The surfaces of a variety of cell types display regional cell surface differences in adhesiveness. For example, the luminal surfaces of endothelial and epithelial cells are apparently non-adhesive under most circumstances. In the teleost embryo, the superficial cells are, like those of the amphibian embryo, non-adhesive at the apical surface and adhesive over lateral surfaces. The yolk mass of blastula and gastrula stage teleost embryos likewise displays adhesive and non-adhesive domains; the internal yolk syncytial layer (YSL) is very adhesive (the embryo proper is situated in contact with the internal YSL), whereas the yolk cytoplasmic layer which invests the portion of the yolk mass not contacted by the blastodisk is demonstrably non-adhesive (Trinkaus, 1971). The superficial cells of cleavage-stage amphibian embryos are, however, a more useful system than the above for study of the mechanisms of non-adhesiveness, since the cells are large and the non-adhesive plaque can be identified in living cells by the underlying cortical pigment granules.

Several mechanisms potentially may contribute to the non-adhesiveness of the apical portions of the superficial cells: (1) A locally high negative surface charge of this portion of the surface might occasion electrostatic forces strong enough to prohibit close approach of the apical surface to other negatively charged surfaces (Curtis, 1967). (2) The macromolecular components external to the plasma membrane may possess a low internal cohesiveness, such that, although these surface components could establish adhesions to other surfaces, the cell could readily be detached from its own surface macromolecules without damage to the cell. (3) The surface may be so rigid that, although adhesion can occur at the points of contact, these areas are not expanded by flattening of the apical surface membrane on to the surface contacted so that the area of adhesion is always too limited to allow the establishment of permanent bonds (Garrod & Born, 1971). (4) A local accumulation at the apical surface of membrane glycopolymers may inhibit contact of surfaces by virtue of their exclusion volume (Maroudas, 1975). (5) Adhesion may be mediated in part by surface macromolecular receptors and ligands (Frazier & Glaser, 1979; Roseman, 1974; Rosen & Kaur, 1979; Vacquier, 1979). The apical surface might lack one or both classes of adhesive macromolecules (Roberson & Armstrong, 1979*b*). (6) Since adhesions appear in many systems to be initiated at the tips of cell protrusions (Lesseps, 1963; Pethica, 1961; Weiss, 1976), the apical surface might remain incapable of initiating adhesions if it were unable to elaborate microvilli (Vasiliev & Gelfand, 1978).

Previous studies have provided evidence in support of mechanism (5): the apical surface of the superficial cells lacks both a surface-located carbohydrate-binding component with oligomannosyl specificity and surface mannose residues (detected by concanavalin A binding) which may be its receptor. Both of these components are found on lateral and basal surfaces (Roberson & Armstrong, 1979*a*, *b*). We have suggested (Roberson & Armstrong, 1979*b*) that adhesion is mediated in part by an interaction between the surface carbohydrate-binding component and oligomannosyl receptors. The absence of these components on the apical surface may contribute to its non-adhesiveness. Mechanism (2) appears not to contribute to non-adhesiveness

in the present system since anion exchange resin beads, which bear a net positive electrostatic charge, adhere permanently to the apical surface. Presumably adhesion is mediated by Coulombic attraction between positively charged bead and negatively charged cell surface. If the glycocalyx at the apical surface was weakly adhesive to the apical plasma membrane, the adhesion between bead and cell would be expected to be temporary, with de-adhesion occurring when that portion of the glycocalyx that adhered to the bead peeled from the plasma membrane. Instead, whenever a bead was mechanically pulled from the embryo, the underlying patch of plasma membrane detached from the cell and remained attached to the bead. The present scanning electron-microscope studies suggest, in addition, that the mechanism suggested by Vasiliev & Gelfand (1978) (i.e. mechanism (6) of the list above) may also contribute to the lack of adhesiveness of the apical surface. Our observations of these cells show that the non-adhesive cell surface region possesses few or no microvilli, while the adhesive surfaces have numerous microvilli. The border between apical and lateral surface regions has the most extensive array of microvilli and it is in this region, in the intact embryo, that the strongest cell–cell adhesion occurs. Consistent also with the proposal of Vasiliev & Gelfand, it is suggested that the microvilli observed in the cleavage furrow serve to initiate adhesion between daughter cells following cytokinesis.

Observations of Trinkaus (1971) and Betchaku & Trinkaus (1978) on microvilli in the developing teleost embryo are consistent with those of the present study and provide support for this hypothesis. In fish, the blastodisk adheres to a specialized portion of the surface of the yolk mass, the internal yolk syncytial layer (YSL). The remainder of the surface of the yolk, the yolk cytoplasmic layer (YCL), is in direct continuity with a band of external YSL which is continuous with the internal YSL. The external YSL and YCL are non-adhesive to glass, carbon particles and embryonic cells, whereas the surface of the internal YSL is highly adhesive to all of these. Indeed, it is downright sticky (Trinkaus, 1971). Significantly, in the light of the observations of the present study, Betchaku & Trinkaus (1978) found that only one of these surfaces is covered with microvilli, that of the internal YSL – the only adhesive surface. The surface of the external YSL is rugose and that of the YCL is quite smooth. Similarly the non-adhesive surface of the superficial cells of the blastodisk are devoid of microvilli on their luminal faces which are, like the luminal faces of the amphibian embryo, non-adhesive (Betchaku & Trinkaus, 1978; Trinkaus, 1971). These surfaces are rugose prior to epiboly and smooth once epiboly has begun. These observations are consistent with the suggestion that non-adhesiveness is dependent on an absence of microvilli; non-adhesive surfaces may be smooth or rugose. In the cleavage-stage teleost embryo, the subsurface cells of the blastodisk are adhesive but without appreciable numbers of microvilli (Trinkaus & Erickson, unpublished observations). This suggests that, in the teleost embryo at least, microvilli may be a sufficient but not a necessary condition for adhesiveness.

The isolated superficial cells appear to be fully viable and are mitotically active, with most cells beginning cytokinesis within 90 min of isolation. Most of the earlier studies on amphibian embryo cleavage involve only the first and second cleavage divisions (Kubota, 1969, 1979; Zotin, 1964; Selman & Waddington, 1955; Singal

& Sanders, 1974; Sawai, 1976). At these stages, the furrow is initiated at the surface of the embryo and proceeds inward. Our observations indicate that isolated superficial cells of mid-cleavage stage (32- to 64-cell stage) embryos display a unilateral or one-sided furrow pattern, with the initiation of the furrow beginning in the basal portion of the cell and not in the apical (pigmented) region. Thus the direction of furrow progression in later cleavage divisions, passing as it does from the basal surface of the superficial cells out to the surface of the embryo, is reversed from the earlier cleavage divisions where the furrow begins at the surface of the embryo. When 32- to 64-cell embryos are subjected to microdissection during a period of cleavage, the pattern of furrow progression is identical to that described for isolated cells, indicating that the cleavage pattern observed in the isolated superficial cells is representative of the cleavage patterns of these cells in the intact embryo. Similarly, cells which are isolated in attached groups of 8–12 cells also display unilateral furrowing with furrow initiation at the basal surface.

It has been suggested that the position of the cleavage furrow is determined by the interaction of a component of the mitotic apparatus with the equatorial cell surface (Rappaport, 1975). Rappaport & Conrad (1963) have demonstrated that unilateral cleavage in *Hydractinia echinata* can be attributed to an eccentric location of the nucleus. In *Hydractinia*, the nucleus is not centred in the cells, but is located much closer to the equatorial surface on the side where the furrow will be initiated. The mitotic apparatus is presumably able to stimulate furrow formation only in that part of the equatorial surface closest to it, with most of the equatorial surface lying too distant for stimulation, resulting in a one-sided or unilateral furrow. In the amphibian superficial cells (32- to 64-cell stage), the nucleus is not located closer to the basal surface than to the apical surface, suggesting that in mid- and late-cleavage stage embryos another mechanism may be involved in the determination of the position of initiation of the cleavage furrow in the superficial cells. Perhaps, by mid-cleavage stages, the apical surface is relatively refractory to the stimulus administered by the mitotic apparatus which initiates furrowing, leading to unilateral initiation at the basal surface.

Our observations of the cleavage furrow region with the SEM indicate an abundance of short, thick microvilli in the cleavage furrow region. These results are consistent with those of Denis-Donini *et al.* (1976), who have shown an increased number of microvilli in the furrow region of intact cleavage stage *Xenopus laevis* embryos. An increased number of microvilli has been demonstrated in the cleavage furrow region in a variety of dividing cells (Gipson, 1974; Arnold, 1969; Knutton, Summer & Pasternak, 1975; Porter, Prescott & Frye, 1973; and Szollosi, 1970). Cell division is accompanied by an increase in surface area. The microvilli in the furrow region may represent sites of stored membrane which is used in the division process. In line with the previous discussion, the microvilli may also be involved in the establishment of adhesive bonds between the daughter cells established by cleavage.

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